MYCOTAXON

Volume 109, pp. 499–512 *July–September 2009*

Glomus custos sp. nov., isolated from a naturally heavy metal-polluted environment in southern Spain

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Abstract — An undescribed species of the arbuscular mycorrhizal (AM) fungi (*Glomeraceae, Glomeromycetes*) was isolated from the bank side of the Rio Tinto River (Huelva, Spain), a naturally heavy-metal polluted environment. The species description is based on spore morphological parameters from in vitro root organ cultures, in vivo pot cultures, in vitro colony morphogenesis, and molecular analyses. Mature in vitro grown and pot cultured spores are pale to brownish yellow, globose to subglobose, 110–172 µm diameter and characterized by 4-layered walls. Phylogenetic analyses of the entire rDNA ITS region place the species into the *Glomeraceae* (group A) without closely related homology with known species. *Glomus custos* forms vesicular-arbuscular mycorrhizae with leek and clover plants under in vivo growing conditions and with excised carrot roots under in vitro propagation. The name *custos* (guardian) refers to the protective properties this fungus confers to host plants in terms of resistance to extreme pH and heavy metal concentrations in soil.

Key words — root organ cultures, molecular phylogeny, new species

Introduction

Spores of a distinct arbuscular mycorrhizal (AM) fungus from a soil sample of a bank of the Rio Tinto River (Huelva, Spain) were isolated and cultures were established under in vitro monoxenic conditions with carrot transformed root organ culture and under in vivo pot-cultures of leek and clover plants. Given that the species population of a given AM fungi community is largely influenced by the land use profile (Li et al. 2007), it becomes important to

identify and characterize a species capable of proliferating in such inhospitable environmental conditions in view of restoration efforts and physiological studies of adaptability of AM fungi.

The Rio Tinto River area is named after the reddish-color of its water, which contains extremely high heavy metal concentrations. When examined, the glomoid spores isolated from the Rio Tinto heavy-metal polluted site differed from published species by their spore wall architecture. Phylogenetic analysis of partial rDNA 18S subunit showed close similarity with group A of the *Glomeraceae* sensu Schüssler et al. (2001) with high homology to *Glomus intraradices* N.C. Schenck & G.S. Sm. 1982. Further analysis of total Internal Transcribed Spacer (ITS) region sequences clearly distinguished *G. custos* from previously described species. Based on morphological and molecular differences, *G. custos* sp. nov. is proposed and described as a new arbuscular mycorrhizal fungi species of the *Glomeromycota*.

Materials and methods

Monoxenic culture of the fungal isolate

Trap plants using natural soil collected from several sites by the Rio Tinto river (Southwestern Spain, Huelva province, 37º42'N/6º36'W) were established and thirty months later spores were isolated from pot cultures (Sieverding 1991), surface-sterilized (Cano et al. 2008), and plated for germination under sterile conditions in a water-agar medium (0.8%, Bacto-Difco agar), and maintained in the dark at 24º C for 10 to 14 days. Germinated spores were transferred (one spore per plate, i.e. monosporic cultures) onto Petri plates containing fresh "minimal medium" (Bago et al. 2004) in which a Ri-T DNA-transformed carrot root organ culture (ROC, DC-2 clone) was vigorously growing. After three weeks, vigorously growing hyphae and branched absorbing structures (BAS) (Bago et al. 1998) began to differentiate, indicating the successful establishment of the Rio Tinto AMF isolate culture. The mother culture was subcultured by transferring plugs of culture medium containing both extraradical mycelium (ERM) and spores to fresh DC-2 ROCs. Tracks of culture traceability have been recorded up to date. Aseptic spores were extracted from in vitro cultures and used to establish pot cultures under greenhouse conditions with *Medicago sativa* L., *Allium porrum* L., and *Lactuca sativa* L. as host plants.

Light and electron microscopy

The spores, from all stages of development, differentiated under in vitro and in vivo cultures were mounted on microscopic slides in polyvinyl alcohol/lactic acid/glycerol (PVLG) (Omar et al. 1979) and in PVLG – Melzer's reagent (1:1, v/v) solution. Terminology of spore characters followed that of Walker (1983) and Stürmer & Morton (1997). Color observations referred to the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM; http://invam.caf.wvu.edu/) color chart codes. Photographs were taken with a Nikon CoolPix 4500 digital camera installed on a Nikon Eclipse 800 compound microscope equipped with

Nomarski differential interference contrast optics. Reference specimens were deposited at the Herbario de la Universidad de Granada (GDA), Spain, the National Mycological Herbarium (DAOM) Canada, the *Glomeromycota* in vitro collection Canada, and the Mycothèque de l'Université catholique de Louvain (MUCL), Belgium.

For transmission electron microscopy (TEM), spores were fixed in a 2.8% glutaraldehyde, 1.5% p-formaldehyde in a 0.2 M phosphate buffer at 4º C for 18 h, washed and centrifuge in a 0.1 M phosphate buffer, and in double distilled water, post-fixed in 2% aqueous osmic acid, dehydrated, stained with uranyl acetate, and embedded in LRWhite (London Resin Company Ltd.). Ultrathin sections were mounted on grids and observed under a Zeiss EM902 electron microscope at 80 kv.

Fungi development under monoxenic cultures

Extraradical mycelium development was observed weekly using a Nikon AFX stereomicroscope with special attention paid to BAS and spore differentiation. Intraradical AM colonization was assessed in selected ROC zones, close to the AMF inoculum source, (Cano & Bago 2006) followed by trypan blue staining (Phillips & Hayman 1970), and mounted in lactic acid on microscope slides and percent root colonization estimated (Trouvelot et al. 1986).

Molecular analyses

PCR analyses on 18S DNA. Approximately 1000 spores were sampled from the monoxenic cultures by dissolving the monoxenic culture medium, according to the method of Doner & Bécard (1991). The DNA extraction followed Declerck et al. (2000) protocol. PCR reactions were conducted on 1/10 of the DNA extract. The following reactives were added: 1x PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl, Invitrogen, USA), 1.5 mM MgCl2, 0.2 µM of each rDNA primer, 200 µM each dNTP (Finnzymes Oy, Finland), 2.5 units of the Taq DNA polymerase (Invitrogen, USA). The two primers used were VANS1: TCTAGTATAATC GTTATACAGG (Simon et al. 1993) and NS8: TCCTCCGCTTATTGA TATGC (White et al. 1990). The amplification was performed in a PTC 200 DNA engine (MJ Research, USA) under the following successive steps: denaturation at 94 °C for 5 min followed by 40 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min with a last cycle at 72° for 7 min. PCR product was cloned in *Escherichia coli* using the Gateway cloning system (Invitrogen, USA) and sequenced using the kit DYEnamic ET terminator cycle sequencing kit (cat n° US 810 50, Amersham Pharmacia Biotech, GB). Clones were sequenced using the following primers NS1, NS2, NS3, NS4, NS5, NS6, NS7, NS8 (White et al. 1990). The sequences were analyzed with an automatic sequencer (CEQ™ 2000 XL DNA analysis system, Beckam Coulter Inc., USA), edited with Sequencher v.4.1.4 (Gene Code Corporation, Ann Arbor, MI), aligned with clustal X 1.5 (Thompson et al. 1997) and corrected manually.

PCR analysis on ITS DNA. The rDNA ITS were amplified with the primers pairs ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'), ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), ITS4 (White et al. 1990), and GLO2A (5'-CGTAACAAGGTTTCCGTAGG-3'), GLO2R(5'-GCGGGTACTCCTAC CTGATT-3'). Precloning PCR was as follow: 3 min at 94°, 38 cycles at 94° for 30 sec, 50° for 60 sec, 72° for 90 sec, with a last cycle of 7 min at 72°. Electrophoresis gels [1.4%

agarose LE (Roche Diagnostics GmbH, Mannheim, Germany) in TAE 1X (Gibco)] were stained with ethidium bromide and photographed under UV light. DNA purification (QIAquick, QIAGEN) and cloning (QIAGEN PCR cloning^{plus} kit, catalogue 231224) were performed following the instructions of the manufacturer on all species for the rDNA ITS and on *G. claroideum* N.C.Schenck & G.S.Sm. 1982 amplified with N4/N629. The sequences were analyzed using 3130XL Genetic Analysers (Applied Biosystems). Between 2 and 8 molecular clones (average of four) of each strain were selected for rDNA ITS and ten of *G. claroideum*. Sequences were aligned with ClustalX v1.83 (Thompson et al. 1997) and manually adjusted with Se-Al v2.0a11 (Rambaut 1996). Aligned sequences were analysed using MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). The number of substitution type was set to 6 (general time reversible model (GTR). Four chains of Markov were run over 300 000 to 1.5M generations for an average standard variation of split frequencies below 0.01. Trees were sampled every 100th generation, and the burning value set to 10%. Dendrograms produced by this Bayesian analysis are shown as 50% majority rule consensus. For both 18S and ITS, sequence homologies were calculated by "BLAST Align 2 sequences" (http://blast.ncbi.nlm.nih.gov/Blast. cgi>).

Taxonomic description

Glomus custos C. Cano & Y. Dalpé, **sp. nov.** Figs 1–19

MycoBank MB 514011

Sporocarpia ignota. Sporae singulae vel laxe gregariae, intra vel extra radices efformatae. Juveniles sporae efformatae in vivo et in vitro, hyalinae vel flavidae, globosae vel subglobosae, 42–90 µm diam., ovoideae vel piriformes, 62–67 × 72–77 µm diam. laevi externa faciei. Juvenilium sporarum tunicae stratis tribus 2.0–4.8 µm crassae; tunica externa hyalina, 0.5–1.0 µm crassa, mucilagina, evanescens; stratum secundum hyalinum, 1.0–1.5 µm crassum laeve, rigidum, aliquando duplo; stratum tertium hyalinum vel flavidum, 1.6–2.8 µm crassum. Maturae sporae efformatae in vivo et in vitro, flavidae vel luteo-brunneae, globosae vel subglobosae, 110–172 µm diam. ovoideae, ellipsoideae, vel tuberculatae, 72–91 × 104–124 µm. Maturarum sporarum tunicae, stratis quatro; tunica externa hyalina, 0.8–2.5 µm crassa, mucilagina, evanescens; stratum secundum hyalinum, 1.6–2.8 µm crassum, rigidum, strato uno adhaerente; stratum tertium hyalinum vel flavidum, semiflexibile, 1.5–2.0 µm crassum; stratum quartum luteum vel luteo-brunneum, laminatum, 2.6–3.8 µm crassum. Hypha sustinente singula, cylindrica vel subinfudibuliforme, 9.6–12.8 µm diam. Porus apertus 1.5–2.6 µm latus, raro septo curvo clausus. Mycorrhizae cum vesiculis et arbusculis formantes.

Holotype. SPAIN, isolated from pot culture at Estación Experimental de Zaidín, Granada, inoculated with a field sample from Rio Tinto, Huelva. GDA 51.596

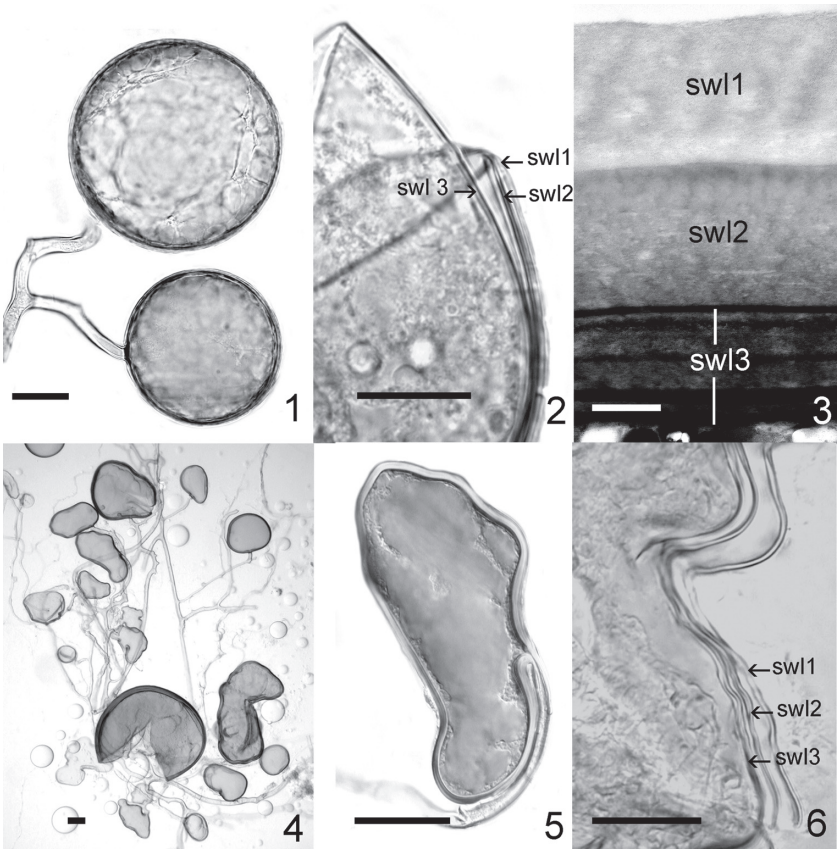
Etymology. Latin *custos*, «guardian, protector» referring to the protective properties conferred by this fungus to the plant host, e.g. superior resistance to extreme pH and heavy metal concentrations.

Sporocarps unknown. Spores formed in roots and soil, singly or in loose clusters of 2–5 spores, under in vitro cultures, spores differentiated distally or intercalary along hyphal ramifications, the distal hyphae rapidly septated and collapsed.

Juvenile spores from 4 months old in vitro cultures and from pot-cultures, established from in-vitro grown spores (Fig.1, 4–5), hyaline to pale yellow, globose, 42–90 µm in diameter, ovoid to pyriform, 42–57 × 72–77 µm in diameter, surface smooth. Spore wall of juvenile spores (Figs 2–3,6) made of 3 detectable spore wall layers, 2.0–4.8 µm total thickness. Outer layer (swl 1) hyaline, 0.5–1.0 µm thick, mucilaginous, evanescent, staining reddish with Melzer's reagent; middle layer (swl 2) hyaline, 1.0–1.5 µm thick, rigid, sometimes double, distinguishable but not easily detachable from layer 1, non reactive to Melzer's; inner layer (swl 4) hyaline to pale yellow, 1.6–2.8 µm thick, laminated with loose arrangement of laminations, only slightly reactive to Melzer's. Subtending hyphae of juvenile spores persistent to spore, straight to slightly flared, (6.4–)9.6–14.4 µm broad at the spore base. Pore open, 6.4–7.2 µm. Subtending hyphal wall, continuous with the three spore wall layers, 3.6–4.8 µm thick at point of spore attachment, decreasing to 1.5–2.0 µm thick at 30–35 µm from the spore. Outer layer (swl 1) thinning shortly, within 5–10 µm along the subtending hypha, inner hyphal wall layers detectable to up to 30 µm along the hyphae.

Mature spores from12 months old in vitro cultures and from pot cultures established from in-vitro grown spores (Figs 7–13), pale yellow (0/0/60/0), to brownish yellow (0/30/100/0), globose to subglobose, 110–172 µm in diameter (mean size 148 µm), sometimes ovoid, amygdaloid to tuberculated, 72–91 × 104–124 µm in size. Spore wall of mature spores made of 4 wall layers. Swl 1 hyaline, mucilaginous, smooth but of irregular thickness, 0.8–2.5 µm thick, reddish with Melzer's reagent, always present on in vitro cultured spores, rarely absent but much thinner with pot culture spores. Swl 2 hyaline (0/0/60/0), rigid, 1.6–2.8 µm thick, outer surface smooth, inner surface occasionally granular on pot cultivated spores, non reactive to Melzer's reagent. Swl 3 hyaline to pale yellow, semi-flexible, surface smooth, easily separated from swl2, 1.5–2.0 µm thick, non reactive to Melzer's reagent. Swl 4, yellow (0/10/80/0) to brownish yellow (0/30/100/0), laminated, 2.6–3.8 µm thick, strongly reactive to Melzer's reagent staining dark red. Spore wall of irregular shaped spores made of 3 detectable layers; swl 3 may be absent similarly to juvenile spores, not reactive to Melzer's reagent in contrast to swl 4.

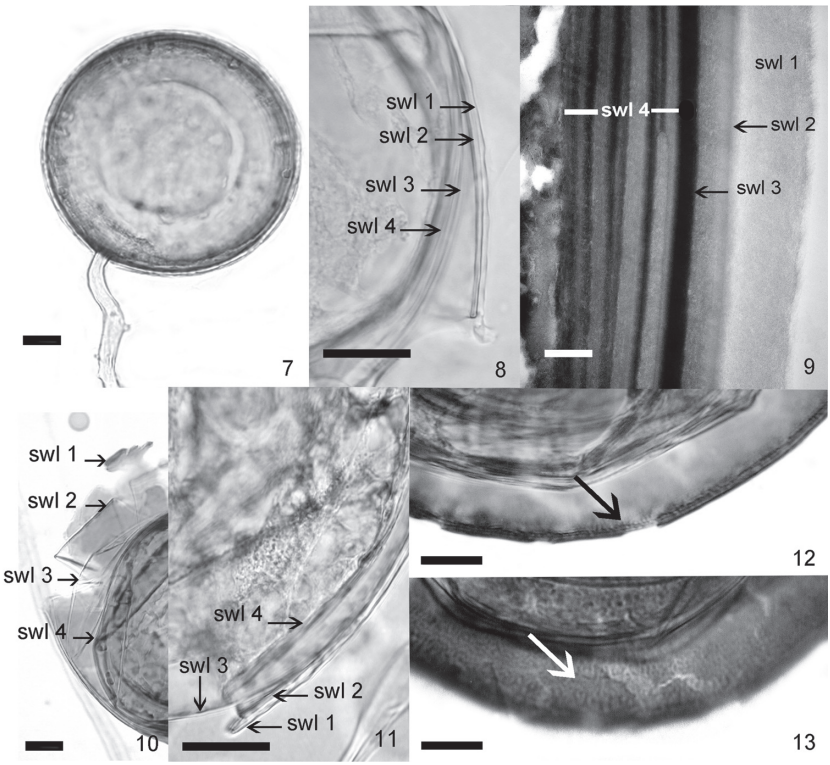
Subtending hypha of mature spores, single, cylindrical, 9.6–12.8 µm in diameter at the spore base, or slightly flared, 12.8–14.4 µm in diameter, concolorous to spore wall. Wall of subtending hypha, 3.2–4.8 µm thick at spore base, made of the 3–4 spore wall layers, swl 1 thinning within the first 5–10 µm from the spore. Pore generally open, 1.5–2.6 µm wide, rarely closed by a curved septum made by the inner laminae of swl 4.



Figs 1–6. Extraradical juvenile spores differentiated in pot-cultures. 1. Globose spores with hyphal attachment. 2. Spore wall layers swl 1,2 and 3. 3. Spore wall layers 1,2 and 4, note clear lamination of swl 4, and regular electron dense layer (arrow) potentially referring to swl 3 of mature spores, not observable under light microscopy. 4. Globose and irregular shaped spores with mycelium, stained with Melzer's reactive. 5. Irregular shaped spore with subtending hyphae. 6. Spore wall layers 1,2 and 3 of irregular shaped spore.

Figs 1,2,4,5,6. Differential interference contrast microscope (DIC). Legend: swl = spore wall layer. Bars = 20 µm. Fig. 3. transmission electron microscopy (TEM), Bar = 1µm.

Intraradical spores from pot cultures, differentiated in loose clusters that can deform root tissue, often disrupting the epidermal cells of root; spores pale yellow to yellowish brown, globose to subglobose, (68–)74–100(–156) µm diam., both juvenile and matures spores found in the same spore clusters. Vesicles (figs 14–15), pale yellow, ovoid to ellipsoid, 30–46 × 60–72 µm in size. Arbuscules (figs 16–17) of Paris-type when cultivated on leek, clover

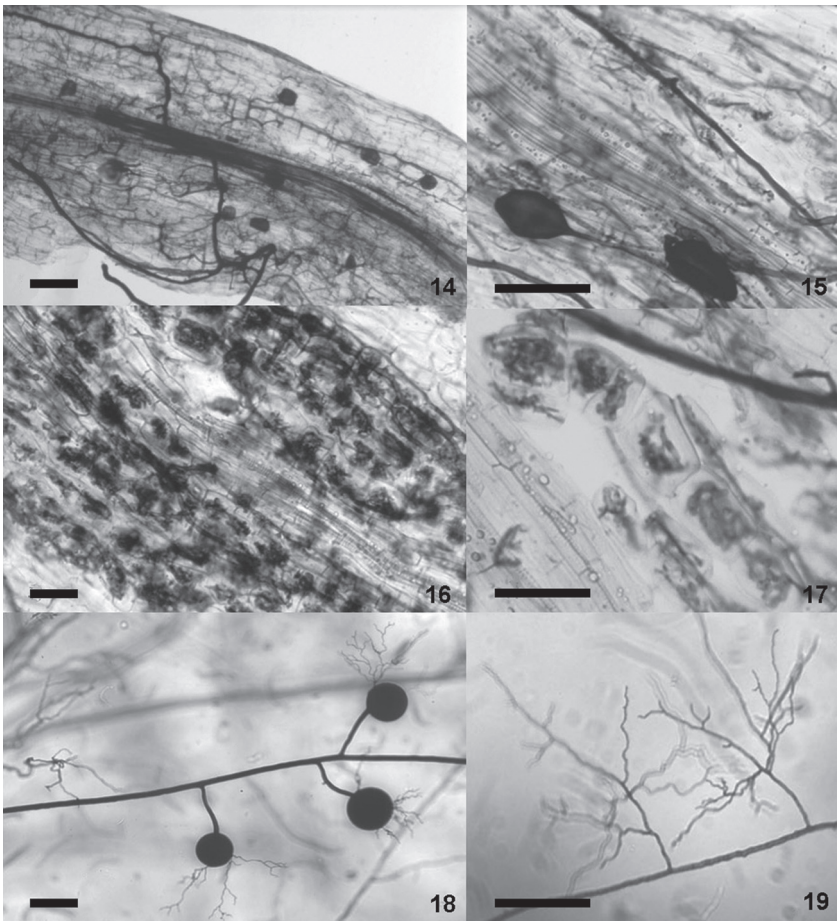


Figs 7–13. Extraradical mature spores differentiated in pot-cultures: 7. Spore with hyphal attachment. 8. Spore wall layers swl1 to 4, mounted in water. 9. Spore wall layers swl l–4, with different gradient density of swl 1 and 2, electron dense swl 3 and lamination of swl 4. 10. Crushed spore stained with Melzer's reactive with non-reactive swl 2 and 3, red staining of swl 1, dark red staining of swl 4. 11. Crushed spore mounted in PVLG with distinct 4 wall layers, inner ornamentation of swl 2 (arrow). 12–13. Spore wall layers stained with Cotton Blue, ornamentation of swl 2 (arrow).

Figures 7,8,10,11,12,13. Differential interference contrast microscope (DIC). Legend: swl = spore wall layer. Bars = 20μm. Fig. 9. Transmission electron microscopy (TEM), Bar = 1μm.

and carrot, often differentiated cell to cell, made of arbusculate coils of deformed hyphae, 3–8 μm diam.

In vitro colony architecture: mycelium made of runner hyphae, 8–12 μm in diameter, sparsely ramified at 45 to 90° angles, 1.4–2.0 μm wall thickness, slightly reactive to Melzer's reagent, ornamented with wart-like excrescences. Branched absorbing structures (BAS, Figs 18–19) located along hyphal ramifications often associated with spore differentiation. Life cycle, from spore germination to differentiation of germinative spores, takes three to four months,



Figs 14–19. Figures 14–17: Fungal structures inside colonized roots. 18–19: Detailed of branching absorbing structures (BAS). 14. Entry points and vesicles along a colonized root, 15. Vesicles. 16. Arbuscular massive root colonization, 17. Arbusculate coils of Paris-type. 18. Spores of the fungal colony formed singly in association with BAS. 19. BAS with well developed branching pattern. Bars = 100µm.

followed by 6–9 months spore maturation process. It comprises an absorptive phase, with ERM covered by BAS, a transition absorptive to sporulative phase, with sporulation starting from the older parts of the fungal colony (closer to the host root) and a sporulative phase with the differentiation of up to 800 spores per cm^3 of gel media.

Mycorrhizal association. Vesicular-arbuscular mycorrhizae formed in pot and in vitro cultures. Field samples from which this species derived had originally

been collected from rhizosphere of a plant consortia consisting of Asteraceae (*Chamaemelum* sp., *Carduus* sp., *Chrysanthemum* sp.), *Boraginaceae* (*Echium* sp.), *Cistaceae* (*Cistus* sp.) and *Poaceae* (*Avena* sp.). In monoxenic culture grown on minimal "M" medium (Chabot et al. 1992) a mean root colonization of 42.0% (±21.8%) was found after 6 weeks of culturing. In greenhouse pot cultures the sp. nov. is maintained with *Allium porrum*, and *Trifolium pratense* L.

Phylogenetic position. The 18S sequence analysis of *Glomus custos* clustered in group A of *Glomeraceae* described by Schüssler et al. (2001) with 99.6% homology with the sequence of *G. intraradices* DAOM 197198. Bayesian sequence analysis of the ITS gene that complements the molecular data is presented as a phylogenetic tree (Fig. 20). The *G. custos* clone ITS sequences group together in a cluster clearly separated from the *G. intraradices* sequence, as indicated by the bootstrap values, with *G. diaphanum* J.B. Morton & C. Walker 1984, the closest related species, grouping on a different branch. When comparing ITS sequence data, the similarity between *G. custos* and *G. diaphanum* reached 82% and Blast alignment indicated between 89 and 97% similarity with an as yet uncultured *Glomus* sp., supporting the status of *G. custos* as a distinct species.

Specimens examined. SPAIN: Rio Tinto, Huelva (pot culture inoculated with soil sample). HOLOTYPE: GDA 51.596; ISOTYPES: GDA 51.597, DAOM 236361, MUCL 47214, consisting of juvenile and mature spores mounted in PVLG and Melzer's reagent.

Other specimens examined. *Glomus aggregatum* N.C.Schenck & G.S.Sm. emend. Koske 1985 (OSC 40250), *G. claroideum* (OSC 40252), *G. gibbosum* Blaszk. 1997 (Blaszkowski collection 10/2051), *G. intraradices* (DAOM 197198),

Distribution and habitat. Originating from a single site at Rio Tinto, Huelva, Spain, 37º42'N/6º36'W, isolated from a pot culture established with rhizospheric soil originating from Rio Tinto, Huelva, altitude 380 m. a. s. l., a dry Mediterranean climate with mean temperatures fluctuating between 17°C in winter and 26°C in summer, and mean annual rainfall of 800 to 900 mm. The rhizospheric soil came from the bank side of the Rio Tinto River, a natural/anthropogenic degraded ecosystem located in a pyritic belt that confers to water and soil a pH of 2 and huge concentrations of Fe (2g/L), Mg (1.3g/L), Cu (390 mg/L), Zn (280 mg/L) and Mn (100 mg/L) amongst other heavy metals. Open-air mines of silver and other appreciated metals have been exploited in the vicinity for 5,000 years, resulting in a highly degraded landscape.

Discussion

Under the dissecting microscope, juvenile spores of *G. custos* closely resemble the spores of *G. aggregatum*, *G. claroideum, G. intraradices*, *G. gibbosum* and

G. irregulare Błasz. et al. 2008 in their hyaline to pale yellow pigmentation, spore diameter range, and hyphal attachment. Under light microscopy, *G. custos* juvenile spores clearly exhibit three spore wall layers that correspond respectively to a mucilaginous evanescent SWL 1, a rigid SWL 2, and the laminated SWL 4 of the mature spores (FIGS 2–3). Similarly as in *G. custos,* the internal spore wall layer of *G. irregulare* is laminated and highly reactive to Melzer's reagent. However, contrary to *G. irregulare*, the outer wall layer (SWL 1) of *G. custos*, is reactive to Melzer's and the middle wall layer (SWL 2) consists of a rigid wall contrary to the semi-permanent, disintegrating one of *G. irregulare*.

The pyriform to tuberculate spores of *G. custos* (FIGS 4–5) differentiate occasionally and only in pot cultures (never in monoxenic in vitro cultures). They closely resemble the irregular shaped spores produced by strains of *G. aggregatum* (Koske 1985, Dalpé 1985) and *G. irregulare* (Blaszkowski et al. 2008). In fact, most *G. custos* spores are globose to subglobose while the majority of *G. irregulare* spores are irregular and rarely globose. Even though differentiated in clusters together with mature globose spores, their spore wall consistently remained 3–layered (FIG. 6). As such, when mature globose spores are absent, these irregularly shaped spores may be confused with those differentiated by *G. aggregatum* and *G. irregulare*. Segregation between species may then require adequate molecular tools. Similarly to *G. aggregatum* and *G. intraradices* species, spores of *G. custos* are often found hidden between thin vermiculite layers suggesting the flexibility of spore walls and the necessity for the fungus to adapt to the restricted space (C. Cano, pers. observation).

The main distinctive characteristics of mature *G. custos* spores are their unique 4-layered spore wall architecture, combined with the red dextrinoid reaction of SWL 1 and SWL 4 to Melzer's reagent (FIGS 7–11), a combined feature not found in any previously published species. Of the 4 wall-layered known *Glomus* spp. with similar spore size range and pigmentation, only *G. claroideum* and *G. gibbosum* spores share some similarity with those of *G. custos*. *Glomus claroideum* spore wall architecture is composed of an outer hyaline mucilaginous SWL 1 often absent in mature spores, slightly reactive with Melzer's and tightly adherent to a semi-flexible hyaline SWL 2, which is non reactive to Melzer's, an outside wall layer arrangement similar to the wall structure observed in juvenile and mature *G. custos* spores. Inner wall layers of *G. claroideum,* however, differ considerably from *G. custos* being made by the succession of a thick laminated wall non-reactive to Melzer's reagent and a flexible innermost layer (http://www.agro.ar.sczcecinagro.ar.szczecin.pl/~jblaszkowski/Glomus%20claroideum.html). *Glomus gibbosum* and *G. claroideum* spores share similar spore wall architecture, except that no *G. gibbosum* wall layer reacts to Melzer's reagent. *Glomus aggregatum, G. intraradices,* and *G. irregulare* — all three-wall layered spores — lack the

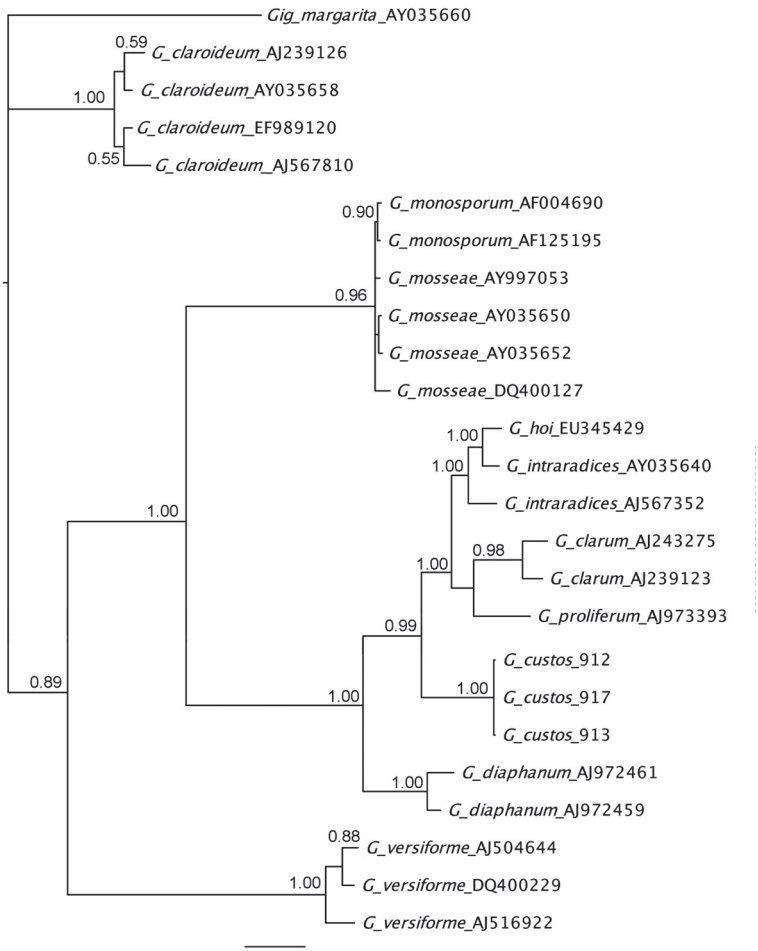


Fig. 20. Phylogenetic tree of rDNA sequences from *G. custos* and other *Glomus* species based on Bayesian analysis of ITS small ribosomal subunit sequences (497 basepairs) *Gigaspora margarita* used as outgroup. Numbers refer to bootstrap values from 1000 replications.

semi-flexible SWL 3 clearly detectable in mature *G. custos* spores (Figs 8–11), their maximum spore size overlaps the smallest *G. custos* spores, and (except for *G. irregulare*) the SWL 3 laminated wall does not react in Melzer's.

The unique spore wall architecture of mature *G. custos* spores allows its segregation from the other described *Glomus* species. However, juvenile

G. custos spores might be confused with spores of species having similar spore wall morphology. As both juvenile and matures spores usually occur together in clusters, the possible confusion should not deter successful identification. An additional *G. custos* spore morphological feature that is likely less valuable (because it occurs on only ~20% of the mature spores) is the granular inner surface ornamentation of SWL 2. This «accessory» feature is more easily detected on Cotton Blue and Melzer's stained crushed spores at high magnification (600×) (FIGS 12–13) and has been observed from both in vivo and in vitro propagated spores. In some mature spores, the inner laminae of SWL 4 may detach and could be interpreted as a separate membranous inner wall layer. Among the 5 wall-layered known *Glomus* species, *G. caesaris* Sieverd. & Oehl (Oehl et al. 2002) differentiates yellow-brown to brown spores with a granular outer wall layer and four successive inner layers (SWL 2–5), a wall architecture different from the one observed with *G. custos* spores.

Phylogenetic analyses of a partial DNA sequence of the 18S ribosomal small subunit gene using in vitro propagated *G. custos* spores place this new species into *Glomeraceae* group A, closely matching *G. intraradices* (DAOM 197198) with a 99.6% sequence similarity. As the species segregation potential of the 18S rDNA SSU gene remains limited, and because spore morphology and spore wall architecture of *G. custos* considerably diverge from those of *G. intraradices*, DNA sequencing of ITS small subunit gene were performed on the same fungal material to complement molecular phylogeny of the strain. ITS analyses revealed a 97% sequence similarity between *G. custos* and *G. intraradices* (DAOM 197198). As seen in FIG. 19, the Bayesian ITS phylogenetic tree clearly separates the *G. custos* clones from its closer related entity, *G. diaphanum*.

Traditional morphological taxonomy, multiple molecular tests, and in vitro propagation technique have allowed the isolation, description, and characterization of *G. custos* sp. nov.

Acknowledgements

The authors want to thank Dr. M. D. Mingorance for the Rio Tinto water and soil analyses, D. Chabot (Agriculture and Agri-Food Canada, Ottawa Canada) for electron microscopy, Dr S. Cranenbrouck (Mycothèque, Université catholique de Louvain Belgium), Dr S. Sokolski (Department of Biology Université Laval Québec Canada), S. Seguin (Agriculture and Agri-Food Canada) for molecular works and analyses, Dr J. Cayouette (Agriculture and Agri-Food Canada) for the Latin diagnosis, and Dr J. Blaszkowski and Dr E. Sieverding for scientific revision. This work was supported by projects AGL2004-06182 (Spanish Ministry of Education and Science). Partial support to C. Cano from REN2003-00968GLO (Spanish Ministry of Education and Science) is also acknowledged.

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